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Serum non-coding RNAs as biomarkers for osteoarthritis progression after ACL injury

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SUMMARY

Objective: The aim of this study was to examine serum non-coding RNAs as potential biomarkers for cartilage damage associated with anterior cruciate ligament (ACL) injury.
Methods: Serum was obtained from 80 patients 1 year after surgery for ACL injury and 60 normal donors without overt skeletal injury. Total serum RNA was isolated, small non-coding RNAs profiled by TaqMan array MicroRNA (miRNA) analysis and individual small RNA assays performed by quantitative TaqMan RT-PCR (qPCR). Semi-quantitative magnetic resonance imaging (MRI) analysis was performed using Whole Organ Magnetic Resonance Knee Score (WORMS) scoring for analysis of cartilage damage.
Results: Initial TaqMan array miRNA profiling showed an increased serum concentration of a small nucleolar RNA (snoRNA), U48, in five patients with cartilage damage compared with that in five patients without cartilage damage and six normal donors. Independent qPCR analysis of snoRNAs in serum from all patients and normal donors showed a strong association between the serum level of another snoRNA, U38, and cartilage damage in ACL injury patients and together with snoRNA, U48, clear distinction between ACL injury patients and normal donors.
Conclusion: SnoRNAs U38 and U48 are significantly elevated in the serum of patients developing cartilage damage at 1 year after ACL injury. Serum levels of U38 have the potential to facilitate early diagnosis of patients with cartilage damage after ACL injury. This study suggests serum non-coding RNAs may serve as novel noninvasive biomarkers for the detection and assessment of cartilage damage after ACL injury.

Introduction

There are currently no effective treatments to prevent osteoarthritis (OA) or slow its progression[1]. Lack of biomarkers able to identify joint pathology before it becomes apparent on radiographs or to predict disease progression has been a major impediment for the development of effective therapies[1]. Previous efforts have focused logically on identifying the products of articular cartilage turnover in serum but have been challenged by many obstacles including: patient and disease heterogeneity; dilution of biomarkers by synovial fluid, lymph and blood; dilution of biomarkers with similar products from other joints and tissues; biomarker degradation; and problems associated with the variability of antibody based assays and the often complex and varied shape of target molecules.

MicroRNAs (miRNAs) are small (approximately 22 nucleotide) RNAs that have received enormous research attention in recent years as overarching post-transcriptional regulators of gene expression[2]. Aberrant expression of miRNAs has been associated with disease development and the recent discovery of miRNAs in serum and plasma stimulated interest in their potential as circulating biomarkers[3]. To date there are more than 100 publications describing circulating miRNA biomarkers of breast, colon, gastric, lung, oral, ovarian, pancreatic, prostate, tongue and squamous cell cancers (see[4–6] for reviews); stroke and neurologic disorders[7], diabetes[8]; heart failure[8] and lupus erythematosus[9]. Serum miRNA biomarkers offer the advantage that they are stable, relatively abundant and their small size and consistent structure enables the application of quantitative multiplex assays using quantitative PCR (qPCR). miRNAs are members of a much larger family of non-protein coding RNAs (ncRNAs) that constitute most of the transcribed genome[10] (only a small percentage of the human genome

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encodes proteins[11]). Other non-coding RNAs include small nucleolar RNAs (snoRNAs)[12], piwi-interacting RNA (piRNAs)[13], long non-coding RNAs[14] and other classes of as yet poorly defined non-coding RNAs[14]. SnoRNAs are a class of small guide RNAs resident in the nucleolus. There are two classes including C/D box snoRNAs (SNORDs) and H/ACA box snoRNAs (SNORAs) associated with the respective methylation and pseudouridylation of ribosomal and some other RNAs[12]. PiRNAs are a very large class of small RNAs present in germ line cells, particularly sperm cells and are believed to function, at least in part, to silence retrotransposons[15]. Long non-coding RNAs are a very large diverse class of RNAs and perform an expanding and wide variety of functions associated with the regulation of gene expression[16]. The presence of ncRNAs other than miRNA in serum and their potential as biomarkers has received very little research attention.

The investigation of serum biomarkers in patients after anterior cruciate ligament (ACL) injury has the advantage that, in contrast to primary knee OA, the development of early degenerative changes can be studied because the duration of the disease, calculated as the time since index injury, is known. The patient population is also more homogeneous, the onset of pathology is relatively rapid and the course of progression of joint pathology is expected to be similar to primary OA[17]. 20–50% of patients develop OA after ACL injury[18] and approximately 12% of all patients with OA can attribute the disease to prior joint trauma[19]. In this manuscript we report the comparison of serum concentrations of miRNAs and snoRNAs with cartilage damage as measured by detailed magnetic resonance imaging (MRI) assessment in patients at 1 year after ACL injury and propose the use of serum snoRNA U38 as a potential biomarker of early cartilage damage associated with post-ACL injury OA.

Methods

Clinical samples

Serum was collected from 102 patients at 1 year after ACL reconstruction as part of the ProKnee.ca study. The study population was drawn from 102 consecutive patients presenting for ACL injury, confirmed by clinical exam and MRI. All patients were 41 years of age or younger without a previous history of knee surgery or systemic health problems that would affect knee OA progression. In all study patients arthroscopic ACL reconstruction surgery was performed within 3 months of the initial injury. Clinical assessment of knee OA pathology was conducted at year 1 post-surgery that included MR imaging evaluation. All MRI examinations were performed on a 1.5 T MR imaging system (Sign Excite HD, GE Healthcare, Waukesha, WI) equipped with high-performance gradients (amplitude, 40 mT/m, slew rate, 200 T/m/sec) utilizing a dedicated eight-channel transmit-receive knee coil (Invivo Corp., Orlando, FL). Imaging sequences performed included a sagittal fast spin-echo (FSE) proton density weighted sequence optimized for evaluation of the menisci, a coronal FSE intermediate weighted sequence, an axial and sagittal FSE T2 weighted sequence with fat suppression, and a sagittal 3D SPGR acquisition with fat saturation. A semi-quantitative evaluation of articular cartilage was made for the medial and lateral tibial plateaus, femoral condyles, as well as the trochlear and patellar articular surfaces using the Whole Organ Magnetic Resonance Knee Score (WORMS)[20]. The total score for articular cartilage was obtained by adding the scores for each of the 16 regions of the joint. Serum was also collected from 60 healthy donors [29 donors were age-matched (within 7 years) with ACL injury patients and 31 were older donors] presenting at Henry Ford Health System for annual physical exam without evidence of skeletal or systemic health problems.

The blood samples from all patients were collected and were allowed to coagulate for 30 min at room temperature and centrifuged for 10 min at 1300*g*. The collected sera were centrifuged further for another 10 min at 3000*g* to remove any remaining cellular component, divided into 500 μl aliquots and stored immediately at −80°C.

Serum RNA isolation

Total RNA was isolated from serum using the miRNeasy mini kit (Qiagen, CA, cat.no.217004) with some modifications. In brief, 400 μl serum was added to 10 volume QIAzol lysis reagent, mixed thoroughly by vortexing and left to stand at room temperature for 5 min. 1/5 volume chloroform was added, shaken vigorously for 30 s, incubated for 5 min at room temperature and centrifuged for 20 min at 12,000×*g* at 4°C. The upper aqueous phase was transferred to a new collection tube, 1.5 volume of 100% ethanol added, mixed thoroughly and loaded onto an RNeasy spin column on a vacuum manifold. The column was washed twice with 500 μl buffer RWT and 500 μl buffer RPE. RNA was eluted by two sequential 50 μl volumes of RNase-free water. The quality of RNA was profiled using NanoDrop 2000 (Thermo scientific, USA).

Reverse transcription

A fixed volume of RNA solution was reverse transcribed using the TaqMan miRNA reverse transcription kit (Invitrogen, USA, Part No. 4366596) using pooled individual miRNA/snoRNA-specific stem-loop RT primers (Applied Biosystems, Foster City, CA). To increase reverse transcription efficiency, a pulsed RT reaction, using the Eppendorf mastercycler (Eppendorf, NY) employing 40 cycles at 16°C for 2 min, 42°C for 1 min and 50°C for 1 s, followed by reverse transcriptase inactivation at 85°C for 5 min was employed. cDNA was used immediately for PCR or stored at −80°C.

MiRNA expression in serum samples from healthy donors and ACL injury patients was profiled using Low Density TaqMan miRNA arrays on a 7900 HT real time PCR System. TaqMan Human miRNA array A and array B (Megaplex RT human pool A and pool B) were used following the manufacture's recommended protocol. The serum RNA was reverse transcribed using the TaqMan miRNA reverse transcription kit and the TaqMan miRNA multiplex RT assays. Each Megaplex RT reaction was followed by one pre-amplification reaction per array with Megaplex PreAmp primers and TaqMan PreAmp master mix. cDNA from multiplex RT reactions was diluted with water and each diluted product combined with TaqMan 2× universal PCR master mix (No AmpErase UNG). 100 μl of the PCR reaction mix was dispensed into each port of the TaqMan miRNA array and qPCR was performed on an Applied Biosystems 7900 HT thermocycler at the manufacturer's recommended cycling conditions. SDS relative Quantification Software version v2.2 was used for data analysis. MiRNA expression was adjusted for small differences in extraction efficiency using global normalization. Applied Biosystems (Life Technologies, CA) instruments, software and reagents were used throughout.

TaqMan qPCR assay

The serum RNA concentration is usually very low, which limits the number of small RNAs that can be measured by qPCR. Pre-amplification enables the RNA/cDNA assayed to be expanded to enhance the number RNAs able to be analyzed and may enhance sensitivity of qPCR. The pre-amplification was performed in a small-scale (10 μl) reaction comprised 5 μl 2× TaqMan PreAm master mix (Applied Biosystems, CA), 2.5 μl pooled individual TaqMan miRNA assay mix (0.2×), 1.25 μl of undiluted RT product

and 1.25 μl of pure water. Pre-amplification was performed using an Eppendorf mastercycler, cycling at 95°C for 10 min, followed by 14 cycles of 95°C for 15 s and 60°C for 4 min, followed by 99°C for 10 min. The pre-amplification PCR product was diluted threefold, mixed and centrifuged briefly.

qPCR was performed in duplicate using the TaqMan qPCR assay, each PCR application reaction was performed in a final volume of 20 μl containing 1.2 μl of cDNA, 10 μl TaqMan 2× universal PCR Master mix, 1 μl miRNA/snoRNA-specific primer/probe mix, and 7.8 μl of pure water. RT-PCR was carried out on an Applied Biosystems 7500 thermocycler, using the following condition: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Raw data were analyzed with SDS relative Quantification Software version 2.2.3 (Applied Biosystems, Inc). The data were normalized to U6 small nuclear RNA using the median procedure[21].

Synthetic single stranded RNA oligonucleotides corresponding to the sequence of U38 and U48 were purchased from Invitrogen (Life Technologies, NY). A series of dilutions of synthetic RNA oligonucleotides were made to generate real time qPCR cycle threshold (Ct) standard curves. The lower limit for detection was designated as the Ct where the linear logarithmic relation with concentration was no longer maintained.

Stability at room temperature

A growing number of studies have shown that serum miRNAs are stable to freeze thawing and can be stored for many weeks at −80°C and at least 24 h at room temperature without loss[3]. 400 μl aliquots of the serum from several individuals was processed immediately and allowed to incubate for extended times (0 h, 1 h, 2 h 4 h and 8 h) at room temperature (25°C) in 2 ml RNase/DNase-free tubes or subjected to four cycles of freeze thawing and immediately processed for RNA isolation. SnoRNAs were analyzed by TaqMan qPCR as described (Supplemental Fig. 2).

Only one study to our knowledge has described snoRNAs in serum or plasma[22]. Our studies of serum snoRNAs confirm their description of snoRNA stability. We did not find any significant change in serum content of U6, hY3, U38 or U48 with storage for up to 8 h at room temperature; freeze thawing for up to four cycles or prolonged storage (6 months) at −80°C. The stability of the snoRNAs was similar to that of miR-16 and miR-197 analyzed in parallel (data not shown).

Statistical analysis

Associations between snoRNA measures and WORMS cartilage score or age were made by Spearman's rank correlations. SnoRNA associations with groups defined by WORMS cartilage score or donor group (normal vs ACL/OA) were assessed by Wilcoxon rank sum tests. The ability of snoRNA copy number to discriminate patients with low *versus* high relative WORMS scores was assessed by receive operator characteristic (ROC) curve construction.

Results

Due to the very low level of serum RNA, we first investigated the specificity and sensitivity of the available PCR-based assays for detecting miRNAs. Two main methods have been available. One is based on miRNAs polyadenylated by poly (A) polymerase and reverse transcribed into cDNA and the other based on stem-loop reverse transcription primers which generate miRNA-specific cDNA templates. In our hands the stem-loop strategy has proven to be robust, highly sensitive and specific for detecting miRNAs. Most importantly, stem-loop based qPCR is based on a newly created template by each individual stem-loop RT primer and this offers unmatched specificity and robust performance for detecting low abundant serum small RNAs, avoiding possible false-positive signals derived from rare contaminated genomic DNA or reagent carry-over.

To investigate the potential of miRNAs as serum biomarkers of OA, array analysis of serum from five patients developing cartilage pathology as determined by detailed MRI analysis (average total WORMS scores 23 range 13–35) was compared with serum from five patients without evidence of cartilage damage at 1 year after ACL injury. Serum miRNAs were also analyzed from six healthy donors without overt skeletal pathology. Approximately 300 miRNAs could be detected in human serum and approximately 150 miRNAs were present in concentrations sufficient to enable reliable measurement, using the TaqMan array.

The serum levels of miRNAs were very similar in patients with and without cartilage pathology or with normal donors (Fig. 1). Those few miRNAs showing different serum levels in patients with and without cartilage pathology were further analyzed using independent TaqMan qPCR assays in serum samples from these and additional patients. The RNA that showed the largest difference in serum levels between patient groups was a non-coding RNA (U48) used as control in the miRNA array. It was also the only RNA whose levels remained significantly different between patients with and without cartilage damage or between patients with cartilage damage and healthy donors on further analysis. To determine if other snoRNAs could be detected in serum and if their

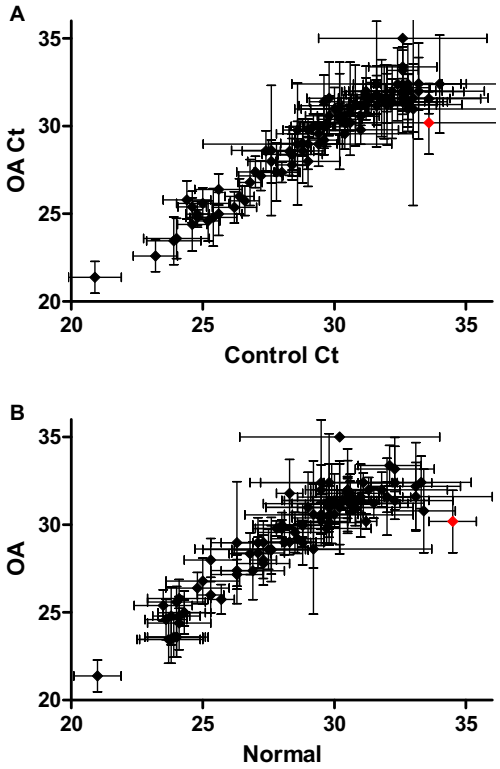

Fig. 1. Comparison of miRNA concentration in serum from ACL injury patients with and without cartilage damage and serum from normal donors. (A) Comparison of miRNA levels in serum from ACL injury patients developing cartilage damage (OA, WORMS score 13–35) and patients without evidence of cartilage damage (Control, WORMS score 0). (B) Comparison of miRNA levels in serum from ACL injury patients with cartilage damage and normal donors. miRNAs were analyzed by ABI TaqMan array after RNA isolation from 0.4 ml serum. Each point represents the average and SD of the Ct of a miRNA from five patients or six normal donors. Points below the normal distribution, such as that shown in red, have higher levels in serum from patients developing cartilage damage. The ncRNA shown in red is snoRNA U48.

concentration correlated with cartilage damage after ACL injury an additional 12 snoRNAs were analyzed. Three snoRNAs in addition to U48 had serum levels consistently above the limits of detection. U24, U48, U49 and U38 all showed a significant increase in serum concentration in ACL surgery patients with cartilage damage compared with our healthy donors. The serum levels of these snoRNAs also showed a tendency to higher concentration in patients with cartilage damage compared to those without cartilage damage; however, the increase was significant for U38 only (Supplemental Fig. 1). The analysis of U24 and U49 was limited by low serum levels, close to the limits of detection and thus not examined further. Analysis of serum levels of U48 and U38 was highly reproducible. Analysis of eleven aliquots of a mixture of serum from several patients generated an average Ct for U48 and U38 of 18.8+− 0.9 [4.7% standard deviation (SD)] and 22.6+− 0.9 (3.8% SD) respectively or copy number of 42,000+− 27,000 (64% SD) and 3600+− 1500 (42% SD), respectively. Normalization with U6 reduced the SD to 2.5 and 2.5% for Ct and 32 and 34% for copy number for U48 and U38 respectively.

Analysis of serum U38 and U48 was expanded to 80 patients at 1 year after ACL surgery and 60 healthy donors, 29 of whom that were age-matched with surgery patients. RNA copy number was determined using standardization with a serial dilution of U38 and U48 RNA oligonucleotides. Patient cartilage pathology was stratified from WORMS scores determined from detailed MRI analysis. U38 levels were below the limits of detection in 28 of the 29 serum samples from normal healthy donors and not significantly different from ACL injury patients without cartilage damage (WORMS score 0) or with minor cartilage damage (WORMS score of 1–3). Patients with greater cartilage damage (WORMS score of $\geq$4) had significantly higher U38 serum levels than either normal donors or patients with minor cartilage damage (WORMS scores below 4) ($P < 0.001$). U48 showed similar trends to U38, although levels in ACL injury patients were not significantly correlated with level of cartilage damage ($r_s = 0.02$, $P = 0.84$). Serum levels of U48 in patients with cartilage damage (WORMS score $\geq$4) were significantly different to that in healthy donors (Fig. 2).

A WORMS score of 3 has been reported in individuals without symptomatic joint pathology[23] and considered here to provide a cutpoint between patients with and without clear articular cartilage damage. Consequently ROC analysis was performed comparing ACL injury patients with WORMS scores up to and including 3 (49 patients) *versus* those above 3 (30 patients). ROC curves of U38 reflected strong separation between these patients with area under the curve (AUC) of 0.743 (95% confidence interval 0.621–0.865) (Fig. 3).

Serum levels of U48 in healthy donors or patients at 1 year after surgery for ACL injury showed no significant change with age between ages 20–75 years ($r_s = -0.17$, $P = 0.20$) or 18–41 ($r_s = -0.09$, $P = 0.51$), years respectively (Fig. 4). The relationship between donor age and serum levels of U38 could not be analyzed for healthy donors because very few serum samples contained detectable U38. There was also no relationship between donor age and serum U38 levels for patients at 1 year after surgery for ACL injury ($r_s = -0.07$, $P = 0.59$).

Discussion

Serum or urine biomarkers of joint degeneration associated with OA have the potential to improve treatment options by providing early diagnosis and facilitating much needed development of new drugs and therapeutic approaches[24,25]. Our studies of

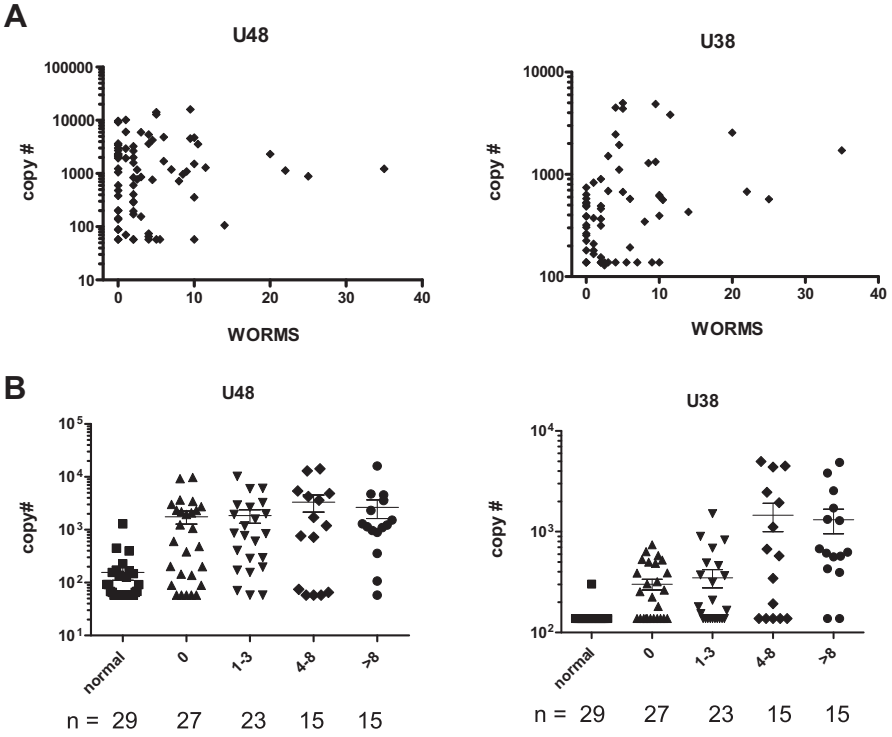

Fig. 2. The association between serum snoRNA U48 and U38 levels and cartilage pathology. The copy number of snoRNAs in serum from patients after ACL injury or normal donors was determined by RT-PCR after reverse transcription. Cartilage damage was determined by detailed MRI using WORMS scoring. (A) Comparison of cartilage damage with patients WORMS score ($r_s = 0.13$, $P = 0.27$ for U48 and $r_s = 0.38$, $P = 0.001$ for U38). (B) The data were binned according to the level of cartilage damage measured by WORMS score for ACL injury patients (0, 1–3, 4–8 and >8). Copy number in age-matched normal donors (normal) is included separately. n = number of donors. For snoRNA U38, serum levels between age-matched control or patients with WORMS scores of 0 or 1–3 and patients with WORMS score >8 all had Wilcoxon $P < 0.05$, For snoRNA U48, all age-matched control comparisons to ACL groups had $P < 0.05$. None of the ACL group to group differences were significant ($P > 0.24$).

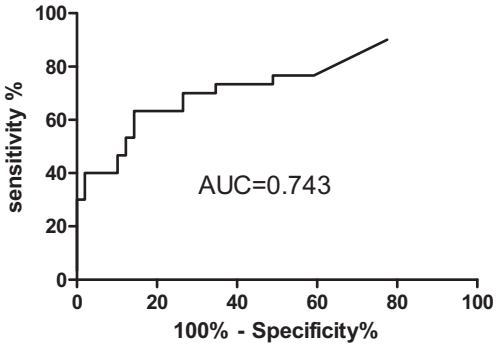

Fig. 3. ROC Plot. The data shown in Fig. 2 were used to draw a ROC plot comparing; U38 serum levels in ACL injury patients with no or very mild cartilage damage (WORMS score ≤3) and patients with greater cartilage damage (WORMS score ≥4).

serum ncRNAs as biomarkers of cartilage damage were stimulated by published studies describing the characterization of serum and plasma miRNAs as biomarkers of several diseases; observations that have grown out of a huge increase in our knowledge of miRNAs and their functions in the last decade. Changes in serum snoRNAs but not miRNAs were associated with cartilage damage in post-ACL injury patients. Previous analyses have shown that miRNAs can be detected in plasma and synovial fluid of patients with rheumatoid arthritis (RA) and primary OA. Plasma levels were generally similar between RA patients, OA patients and healthy controls except miR-132 that was shown to have significantly lower levels in the RA and OA patients compared with healthy controls[26]. In our array studies the levels of miR-132 were similar and close to the limits of detection in serum from patients with or without cartilage damage at 1 year after ACL injury and in our healthy donors. We did not analyze miRNAs or snoRNAs in synovial fluid from any donors or in serum from patients with primary OA. SnoRNAs (and other ncRNAs), like miRNAs, are emerging as important regulators of cell function and disease development. Mutations in snoRNAs (SNORD 115 and 116) have been shown to cause a neuro-developmental disorder, Prada-Willi syndrome[27]; snoRNA U50 has been shown to be a tumor suppressor gene down regulated in breast and prostate cancer[28] and SNORA 42 has been shown to be associated with non-small-cell lung cancer[29]. Other studies have shown that loss of three snoRNAs, U32a, U33 and U35, is sufficient to confer resistance to cell death induced by oxidative stress *in vitro* and *in vivo*[30]. Furthermore, current studies, primarily conducted in yeast, suggest that snoRNAs fine-tune the ribosome to accommodate changing requirements for protein production during development, normal function and potentially diseases such as cancer[31].

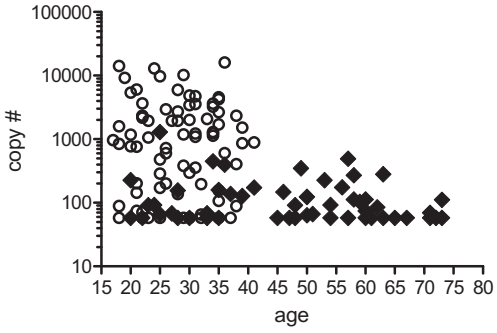

Fig. 4. Age association of serum concentration of U48. The serum concentration of U48 (copy #) from ACL injury patients (open circles) or normal donors (black diamonds) is plotted against the age of the donor.

It is becoming increasingly evident that the regulation of ribosome function and activity (and by analogy snoRNA expression) plays a pivotal role in regulating the function of high protein producing cells such as, chondrocytes and other connective tissue cells, and is reflected in the phenotypes of ribosomophathies[32]. Emerging data also indicate that the nucleolus responds acutely to cell stress which can stimulate p53 expression, apoptosis[33,34] and autophagy (piecemeal microautophagy or nucleophagy)[35].

The detection of snoRNAs in serum was surprising since they are normally resident in the nucleolar subcompartment of the nucleus and except for one publication[22] have not been described in the circulation. Consistent with the previous report our studies have shown that snoRNAs are very stable in serum. Our preliminary studies (data not shown) suggest that, like the bulk of miRNAs, serum snoRNAs are not present in microvesicles or exosomes but are present as stable, unidentified protein complexes. Several mechanisms for the release of miRNAs from cells and into the circulation have been proposed. These include exocytosis in microvesicles or exosomes[36], release as protein complexes[37,38] or the consequence of cell death[39]. Cell to cell transfer of functional miRNAs has been demonstrated in culture and has been proposed for circulating miRNAs[6]. The mechanism of release of snoRNAs has not been reported, however; apoptosis and modulation of the normal, autophagic process can result in the release of cell components including those of the nucleus and nucleolar compartment[40].

The development of biomarkers for post-traumatic OA has the distinct advantage over studies of primary OA that the time of onset of the disease (injury to the ACL in the present studies) and thus duration of the disease is known. In this study detailed clinical, functional and MRI assessment of the entire knee joint allowed stratification of ACL injured patients and early diagnosis of subtle cartilage damage consistent with the early OA. The association between the cartilage damage analyzed and the serum levels of U38 suggests analysis of U38 in serum can provide a biomarker of early events in joint degeneration. The elevated levels of U38 and U48 in serum of ACL injury patients without visible cartilage damage compared with normal donors suggests serum levels of these snoRNAs might be associated with the initial injury. However; distinct differences in serum levels of U38 between patients with and without cartilage damage after ACL injury suggests the serum levels of U38 are associated with early events in joint degeneration rather than the consequence of the initial injury. Also the absence of changes in serum U38 (or U48) with age in our normal donors suggests that serum U38 is not associated with normal aging or age-related cartilage degeneration, which takes place more slowly. The highest levels of U38 in serum were found in patients with relatively low WORMS scores (5–10) suggesting release of U38 might be associated with events earlier in joint degeneration than are readily detected by MRI analysis. Whether the serum levels of U38 and U48 are prognostic of further cartilage damage will be determined when serum and clinical analysis from follow-up studies at years 2–5 after ACL injury become available.

Our analysis included only a very small proportion of the estimated approximately 500 snoRNAs expressed in the human genome[41]. Whether other snoRNA biomarkers of cartilage damage are found will require sensitive array analysis or sequencing of serum RNAs. The link between serum U38 and cartilage damage remains to be determined. At present the source of the serum snoRNAs is unknown; however, it seems unlikely that these snoRNAs come from damaged articular cartilage. Although snoRNAs, like miRNAs, are stable in serum and are not as susceptible as proteins or carbohydrates to enzyme digestion, they would be expected to be similarly diluted by synovial fluid and blood such that snoRNAs released from a small number of damaged chondrocytes

would be undetectable by the time they reached the peripheral circulation. RNA sequencing studies have shown substantial tissue specificity in snoRNA expression. U38 concentrations have been shown to be 20–100 fold greater than U48 in all tissues studied (heart, liver, kidney, skeletal muscle, brain, adipose, colon, ovary, testes and spleen, connective tissues were not analyzed)[42] in marked contrast to the analysis of their relative serum levels in the present studies. This might suggest that U38 and U48 came from tissues not analyzed previously or that specific snoRNAs are secreted under certain conditions. SnoRNAs have the advantage that serum levels are largely unaffected by hemolysis (a problem in serum miRNA analysis) due to the absence of the nucleolus and nucleus in erythrocytes.

In conclusion our studies have shown that the levels of snoRNAs can be analyzed in serum and that the level of U38 and U48 is elevated at 1 year after ACL injury. Furthermore the serum levels of U38 are substantially elevated in those patients who develop cartilage damage after ACL injury suggesting snoRNA U38 is a serum biomarker for early cartilage damage.

Author contributions

LZ, MY and GG developed and designed serum RNA analysis and prepared the manuscript. GD provided the statistical analysis and contributed to manuscript preparation. MH, LMW and PM designed and conducted the post-ACL injury study and patient assessment. All authors read and approved the final manuscript.

Conflicts of interest

None of the authors have competing interests.

Acknowledgments

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Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2012.08.016.

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